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M-band: a safeguard for sarcomere stability?

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Abstract

The sarcomere of striated muscle is a very efficient machine transforming chemical energy into movement. However, a wrong distribution of the generated forces may lead to self-destruction of the engine itself. A well-known example for this is eccentric contraction (elongation of the sarcomere in the activated state), which damages sarcomeric structure and leads to a reduced muscle performance. The goal of this review is to discuss the involvement of different cytoskeletal systems, in particular the M-band filaments, in the mechanisms that provide stability during sarcomeric contraction. The M-band is the transverse structure in the center of the sarcomeric A-band, which is responsible both for the regular packing of thick filaments and for the uniform distribution of the tension over the myosin filament lattice in the activated sarcomere. Although some proteins from the Ig-superfamily, like myomesin and M-protein, are the major candidates for the role of M-band bridges, the exact molecular organisation of the M-band is not clear. However, the protein composition of the M-band seems to modulate the mechanical characteristics of the thick filament lattice, in particular its stiffness, adjusting it to the specific demands in different muscle types. The special M-band design in slow fibers might be part of structural adaptations, favouring sarcomere stability for a continuous contractile activity over a broad working range. In conclusion, we discuss why the interference with M-band structure might have fatal consequences for the integrity of the working sarcomere.

General aspects of sarcomere stability

Eccentric contraction

Passive skeletal muscle can be stretched or compressed without any effect on its contractile properties. However, the elongation in the activated state, called eccentric contraction, leads to an impaired performance of the muscle fibers (reviewed in Allen and Leinwand, 2001; Proske and Morgan, 2001). For example, downhill walking with a backpack leads to the eccentric loading of weight-bearing muscles which, if untrained, become stiff and sore for many days.

Muscles subjected to eccentric contraction display two prominent signs of damage: disrupted sarcomeres and impaired excitation–contraction coupling (Proske and Morgan, 2001; Warren *et al.*, 2001). The 'popping sarcomere' hypothesis is based on the assumption that muscle activation in the extended state leads to regions with irregular sarcomere length (Morgan, 1990). It proposes the following scenario for damage during eccentric contraction: first, the weakest sarcomere starts to elongate uncontrollably until the acto-myosin overlap is completely lost in one-half of the sarcomere. This leads to a hypercontraction of the opposite half-sarcomere (Macpherson *et al.*, 1997) and the resulting shearing stress breaks the membranes of structures that are involved in excitation–contraction coupling (Yeung *et al.*, 2002). The subsequent leakage of ions causes impaired excitation–contraction coupling and might even result in inflammation following eccentric exercise (Allen, 2001).

This hypothesis describes the consequences of a single eccentric contraction and successfully explains many experimental data. However, it fails to answer the question why the loss of acto-myosin overlap in individual sarcomeres, which are supposed to reinterdigitate during muscle relaxation, leads to expanded regions of disrupted sarcomeres that are observed in the myofibers after repeated eccentric exercise (see for example Ogilvie *et al.*, 1988). The idea proposed here (discussed in more detail below) is that the weakening of the overstretched sarcomere might be due to the progressive damage of the stabilizing systems, in particular, the titin filaments.

Several filament systems and cytoskeletal elements stabilize the sarcomere

To achieve an effective and stable contraction the length of all sarcomeres should be restricted to the region of the plateau in the length-tension relationship (Morgan, 1990). For this, two conditions must be satisfied: first, the total length of a myofibril has to be limited and second, the sarcomeric lengths should be distributed evenly. Usually, non-muscle elements, such as ligaments,

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tendons and bones provide the physiological constraints for maximal muscle stretch in skeletal muscle. These structures are not present in heart muscle; therefore, it needs the reinforcement of some other structures to prevent overstretching. Interestingly, a recent modelling study suggested that the fiber orientation in the ventricular wall is optimized to minimize variations in sarcomere length (Vendelin *et al*., 2002).

Different elastic and cytoskeletal systems, involved in the force transmission (for review see Patel and Lieber, 1998) might also contribute to regulate sarcomere length. Most important among these are titin filaments, intermediate filaments and the extracellular matrix (ECM). These filament systems work at different levels in the muscle cell. Up to now, only the contribution of these elements to passive muscle stiffness has been analyzed and it is currently difficult to estimate their mechanical characteristics in the dynamic situation of the activated sarcomere. However, mutational or protein-knock-out studies in transgenic animals bring some insights into this issue.

Within the sarcomeres the major contribution for length stabilization is thought to come from the elastic titin filaments (for review see Gregorio *et al*., 1999; Granzier and Labeit, 2002; Tskhovrebova and Trinick, 2002). The giant titin molecules overlap in the Z-disc and M-band of the sarcomere, forming a continuous elastic filament system inside the myofiber. Thus, it was assumed that titin is the main structural element responsible for the even distribution of sarcomeric length in non-activated muscle (Goulding *et al*., 1997). The extensible I-band region of titin consists of stretches of Immunoglobulin-like (Ig)-domains, separated by the unique PEVK and N2B segments. These structural elements are characterized by different elasticity and are sequentially recruited during stretch (Linke and Granzier, 1998; Linke *et al*., 1999; Trombitas *et al*., 1999). Only a single copy of the gene has been identified in the vertebrate genome, while a number of distinct titin isoforms is generated by alternative splicing (Kolmerer *et al*., 1996). Different isoforms of the I-band portion of titin are involved in controlling the elastic properties of different muscles (Freiburg *et al*., 2000), while other isoforms correlate with Z-disc (Gregorio *et al*., 1998) and M-band heterogeneity (Labeit and Kolmerer, 1995). Lack of titin or expression of titin truncations either prevent myofibrillogenesis or lead to severe myopathies and premature death, underlining the crucial importance of this molecule (van der Ven *et al*., 2000; Xu *et al*., 2002).

Costameres are rib-like sarcolemma-associated multiprotein complexes, which are characterized as mechanical couplers distributing the contractile forces generated in the sarcomere to the basal lamina (Pardo *et al*., 1983; Danowski *et al*., 1992). The costameres might be the key elements, which integrate the intracellular and extracellular filament systems involved in sarcomere stabilization (Anastasi *et al*., 1998; Monti *et al*., 1999). Neighbouring costameres are connected by filaments, which lie in the submembrane region and consist mostly of dystrophin, spectrin and associated proteins (Williams and Bloch, 1999). It is believed that the dystrophin glycoprotein complex is also responsible for the mechanical link to the ECM, composed of collagen, fibronectin, laminin and a significant number of other proteins. The ECM is an important element of the sarcomere stabilization system (Huijing, 1999), especially in cardiac muscle (Hanley *et al*., 1999). While the role of the costameres during eccentric contraction is still poorly understood, it has been found that mutations in proteins involved in these membrane contacts such as dystrophin, sarcoglycan, vinculin and laminin result in the impaired management of contractile stress and might finally lead to dilated cardiomyopathy (DCM) and muscular dystrophy (Hack *et al*., 2000; Towbin and Bowles, 2000; Nishino and Ozawa, 2002).

The link between the costameres and the sarcomeres is provided by the intermediate filament system, which consists mainly of desmin in striated muscle. Desmin filaments are concentrated around the Z-disk and in addition run laterally along the myofibrils (Street, 1983; Wang and Ramirez-Mitchell, 1983). While knock-out mice for desmin are viable, their muscles show a significantly reduced resistance to mechanical stress (Thornell *et al*., 1997) resulting in cardiomyopathy at later stages and in dystrophy of continuously used muscles, such as soleus and diaphragm (Li *et al*., 1996, 1997; Milner *et al*., 1999). Mutations in the desmin gene have also been associated with hereditary forms of DCM and myopathies in humans (Carlsson *et al*., 2002; Carlsson and Thornell, 2001).

Repeated eccentric contraction might overload the sarcomere stabilization capacity

The disruption of sarcomeric structures following eccentric contraction might be caused by mechanical overloading and progressive damage of the stabilizing cytoskeletal systems. This suggestion is supported by observations that the staining of desmin (Lieber *et al*., 1996; Friden and Lieber, 1998) and of dystrophin (e.g. dystrophin-associated proteins) is lost following eccentric exercise (Biral *et al*., 2000). Recent progress in the understanding of the mechanical properties of titin suggests that overstretching by eccentric contraction might affect its elastic region too. The unfolding of titin Ig-domains was first characterized by *in vitro* stretching of individual titin molecules (Tskhovrebova *et al*., 1997), but for a long time it was believed that this process does not take place in muscle *in situ* during stretching within the physiological limit (Linke *et al*., 1999; Trombitas *et al*., 1999). However, a recent study concluded that the Ig-unfolding event becomes relevant already for a sarcomeric length of about 2.6 μm, which corresponds to an elongation of individual titin strands by only 200 nm (Minajeva *et al*., 2001). This indicates that the unfolding of Ig-domains is a stochastic event with no well-defined threshold force and that the probability of

unfolding increases continuously with stretching (Rief *et al.*, 1998). If the myosin filaments lose their overlap with actin on one side of some sarcomeres during eccentric contraction, as suggested by Morgan (Morgan, 1990), the strain on the titin molecules on this side would far exceed the physiological limits and would probably result in the unfolding of many Ig-domains.

During muscle relaxation stress ceases, allowing the disrupted titin domains to fold again. However, the problem is that the refolding of Ig-modules of titin is a relatively slow process with an estimated rate up to 1 s^{-1} (Carrion-Vazquez *et al.*, 1999; Minajeva *et al.*, 2001). If several domains were unfolded, the complete recovery would take seconds (Kellermayer *et al.*, 1997). Thus, if the next contraction cycle begins before this process is completed, titin with a still extended contour length would not manage to center the A-band properly and the same half of the sarcomere will be weakened and again overstretched. It was shown that the repetition of the refolding–unfolding process might even lead to an irreversible denaturation of some titin Ig-domains (Kellermayer *et al.*, 1997). This scenario is supported by the observation that muscles subjected to eccentric contraction show a more intense staining by an anti-titin antibody (Lieber *et al.*, 1996), which might be explained by the increased accessibility of the epitope in the unfolded titin domains. Alternatively, recent single molecule manipulations suggest that in addition to the unfolding processes, mechanical fatigue of titin molecules might result from a rupture of some non-specific bonds that cross-link parts of the titin molecule in the relaxed state. While the exact nature of these interactions remains unclear, a relaxation time in the order of minutes is needed for complete recovery (Kellermayer *et al.*, 2001). In conclusion, molecular fatigue in repetitively stretched titin molecules might lead to an overall elongation of the affected sarcomeres. This in turn would overload the other cytoskeletal stabilization systems, leading to a further propagation of the defect along and across the muscle fiber by repeated eccentric contraction (Allen, 2001).

M-band bridges are essential for stable contractions of the sarcomere

M-band (or M-line) is the name of the structure that appears as a series of dark transversal lines in the center of the sarcomeric A-band. Similar to Z-discs that anchor actin filaments, the M-band bridges ensure the regular packing of myosin filaments and are very important for sarcomere stability during contraction. Despite the fact that a number of proteins, localized in this region of the sarcomere have been already characterized, the molecular organization of the M-band is not clear. In this part of the review we propose an important functional role of the M-band in the sarcomere and discuss how this structure adapts to specific mechanical demands in different muscle types. In conclusion, we speculate why any interference with the M-band structure might lead to gradual sarcomere damage.

The M-band might be responsible for the regular packing of the thick filaments

Five prominent lines constituting the sarcomeric M-band can be distinguished by electron microscopic analysis of longitudinal sections of myofibrils: M1, M4/M4′ and M6/M6′ (Sjostrom and Squire, 1977). The M-band appearance correlates with the physiological performance of a particular skeletal muscle type. In general, the fastest fibers have a 3-line pattern, while the slowest fibers have 4-lines and fibers of intermediate speed have variations of a 5-line pattern (Sjostrom and Squire, 1977; Carlsson and Thornell, 1987; Edman *et al.*, 1988). The M-band pattern in cardiac sarcomeres correlates with the heart rate in a given species (Pask *et al.*, 1994). In addition, the appearance of the M-band can change during development, for example the central M1 line disappears during the first four weeks from the differentiating slow fibers (Carlsson and Thornell, 1987). These observations indicate that the M-band is a dynamic structure, which reorganizes in respond to the change of contractile parameters.

Cross-sections of this region of the sarcomere reveal that thin lines, called M-bridges, connect the myosin filaments to each other (see Figure 1). High resolution images of negatively stained M-bands showed also the existence of so called M-filaments running in parallel to the myosin filaments in some muscle types. These M-filaments, in turn, are joined by secondary M-bridges at

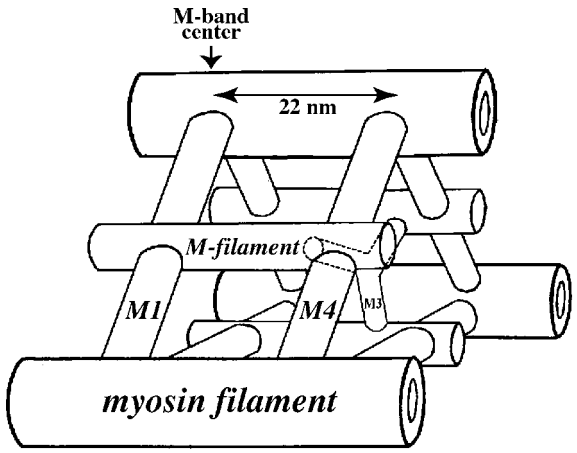

Fig. 1. Schematic representation of the M-band model proposed by Knappeis and Carlsen (1968) and revised by Luther and Squire (1978). The two M-bridges, corresponding to the most prominent lines observed in EM-pictures, are designated M1 and M4 (Sjostrom and Squire, 1977). In a three-line M-band, the structure as visualized here would be mirrored in the M1 plane to yield the whole M-band assembly. The so-called M-filaments run in parallel to the myosin filaments in some muscle types. The Y-shaped elements are supposed to be the secondary M-bridges, joining the M-filaments at the M3/M3′ lines. Despite a lot of effort, the molecular composition of the M-band structural elements is not clear yet. Adapted from Luther and Squire (1978).

the M3/M3′ lines. These observations gave rise to the first 3-D model of the M-band ultrastructure (Luther and Squire, 1978).

According to its location in the sarcomere, the M-band was suggested to be responsible for the regular packing of thick filaments (Luther *et al*., 1981). Although rather elastic, the M-bridges might prevent excessive deformations of the lattice in the relaxed sarcomere, providing the optimal distance between thick and thin filaments at the onset of contraction (Millman, 1998). Moreover, the M-band proteins might play an important role during myofibrillogenesis, when the nascent thick filaments have to be integrated with the titin filaments and packed into a regular hexagonal lattice (Ehler *et al*., 1999).

The dynamic role of the M-band in the activated sarcomere

One of the consequences of the sliding filament model of muscle contraction is the intrinsic instability of the thick filament position. The actin filaments do not have a uniform length within a sarcomere (Robinson and Winegrad, 1979; Traeger and Goldstein, 1983). If one half of the activated thick filament has a slightly larger overlap with the actin filaments, the force acting in this direction will be increased, displacing thereby the myosin filaments further from the center (see Figure 2).

According to the current concept, the elastic titin filaments, which link the myosin filaments with the two adjacent Z-disks, are counterbalancing this effect by supplying a centrally directed restoring force (Squire, 1997; Patel and Lieber, 1998). However, recent measurements on titin elasticity revealed that the force generated by titin might not be strong enough. Taking into account the sarcomere length of 2.2 μm, a myosin filament deviated up to one Z-disc stretches the titin molecules, attached to the opposite side, by about 0.3 μm. Single-molecule experiments have shown that at such elongations titin behaves as a very compliant spring that is able to produce a weak force of about 4 pN (Rief *et al*., 1998; Li *et al*., 2002), which is comparable with the force generated by one myosin head (Ishijima *et al*., 1996). In contrast, the axial force, exerted by a deviated thick filament might approach hundreds of pN. Therefore, even six titin molecules together (Liversage *et al*., 2001; Knupp *et al*., 2002) cannot compensate for it. Thus, during cross-bridge activation the neighbouring myosin filaments might accidentally slide in different directions, breaking the A-band alignment. A pioneering experiment showed that the A-bands indeed deviate from their central positions during long isometric contraction (Horowits and Podolsky, 1987). In electron micrographs it is apparent that this is not the case for individual thick filaments but that the whole A-band with a well-defined A/I boundary and a distinct M-band is displaced in the direction of one of the Z-discs.

This suggests that there might be a division of labour between two kinds of filaments in the sarcomere: the M-band proteins are responsible for the perfect lateral alignment of thick filaments during the activation, while titin filaments provide the centering of the A-band as a whole and brings it back to a central position during muscle relaxation (Figure 2). Further, in a system where thick filaments are firmly attached to each other the random component of the force unbalance is reduced by a factor equal to square root of their number (Meyer, 1966). Thus, the M-band structure might promote the symmetric shortening of both halves of the sarcomere by smoothing out differences of cross-bridge forces over all thick filaments present in one A-band. This proposed role of the M-band implies that its mechanical properties should depend on the contractile parameters in a given muscle, which are mainly determined by isoforms of myosin. This might be reflected by a diverse molecular composition of M-bands in different muscle types.

Structural components of the M-band

In contrast to the other transverse sarcomeric structure, the Z-disc, where a plethora of proteins has been identified (for review see Faulkner *et al*., 2001), comparatively few M-band components have been found so

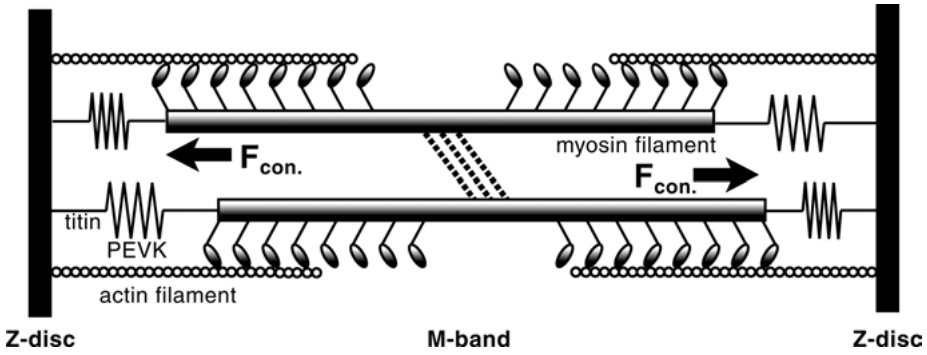

Fig. 2. Elastic titin filaments and the M-band play different roles in the activated sarcomere: during sarcomere activation the thick filaments try to escape into different directions, according to differences in the amount of activated cross-bridges on both halves. The M-band filaments equilibrate these imbalances in force through all thick filaments in the half-sarcomere ensuring therefore a symmetrical shortening of the sarcomeric unit. The titin filaments are responsible for the centering of the A-band unit as a whole.

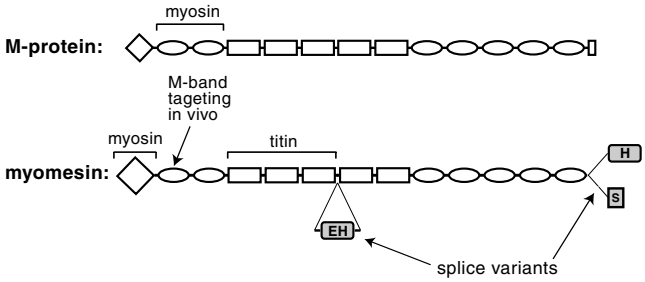

Fig. 3. Schematic representation of the two M-band components, M-protein and myomesin: they consist mainly of immunoglobulin-like (ellipses) and fibronectin type III domains (rectangles) in a similar arrangement with the major heterogeneity residing in their unique N-terminal head domains. Another difference is the existence of splice isoforms in the case of myomesin. A splicing event at the C-terminus leads to the differential expression of H (heart)- and S (skeletal)-myomesin isoforms in the corresponding avian muscles. In contrary, mammalian muscles express S-myomesin in all muscle types. The EH (embryonic heart)-myomesin isoform is generated by the inclusion of the EH-fragment in the middle part of the molecule. It is the main myomesin isoform in the embryonic heart of all higher vertebrates and is weakly expressed in slow fibers of adult mice. Already identified binding sites for myosin and titin in both M-band components are shown by brackets above the molecules.

far. In addition to the C-terminal part of the titin filaments and the myosin heavy chain tails, there are the structural components myomesin and M-protein. Both seem to be closely related, suggesting a common ancestor (Kenny *et al.*, 1999) and share a similar domain composition with a unique head domain followed by a sequence of Ig and fibronectin type three domains (Figure 3). Both myomesin and M-protein have been shown to bind to titin and myosin, supporting their role as integral structural components of the M-band. However, in contrast to myomesin, which is expressed in all types of vertebrate striated muscle investigated so far, and becomes localized in an organized fashion in the nascent sarcomeres (Ehler *et al.*, 1999); M-protein displays a developmental stage and fiber-type regulated expression pattern (Grove *et al.*, 1989; Carlsson *et al.*, 1990). In heart, no M-protein could be detected before birth and upregulation of its expression occurs only postnatally. In skeletal muscle on the other hand, M-protein is expressed during embryonic development but is down regulated specifically in slow twitch fibers in the first two weeks after birth. This suggests that myomesin might play a more general role as an integrating molecule in the M-band while M-protein serves for the fine-tuning of M-band properties in some muscle types.

Myomesin isoforms

Another difference between the two M-band components is the existence of isoforms in the case of myomesin (see Figure 3). The first indication for isoform diversity came from the finding of myomesin bands with different molecular weight in chicken heart and skeletal muscle that originate from different transcripts (Grove *et al.*, 1985; Bantle *et al.*, 1996). The corresponding myomesin isoforms, designated H (heart)-myomesin and S (skeletal)-myomesin, represent the major myomesin species in heart and skeletal muscle of chicken, respectively, while in mammals the S-myomesin isoform is expressed in both heart and skeletal muscle (Agarkova *et al.*, 2000). The EH (embryonic heart)-myomesin isoform, is generated by the insertion of an additional fragment, the EH-fragment, in the middle of the molecule between domains six and seven (Figure 3). Sequence comparison revealed that the EH-fragments of chicken, mouse and human are quite heterogeneous, so that antibodies generated against the chicken fragment barely cross-react with EH-myomesin of other species. Computer simulations predict an increased flexibility of the EH-fragment compared to the immunoglobulin domains that make up the larger part of the myomesin molecule. In accordance to this, the circular dichroism spectrum of this fragment corresponds to a largely unfolded protein (I. Agarkova, unpubl.). It was suggested that the EH-fragment could function as a flexible elastic stretch in the middle part of the myomesin molecule. In this sense, EH-myomesin resembles a miniature of titin with the EH-fragment being the analog of the PEVK fragment. This longer myomesin isoform is expressed in the embryonic heart of all higher vertebrates (Agarkova *et al.*, 2000) and to a reduced extent in slow fibers of adult mice. Interestingly, EH-myomesin appeared to be expressed in a reciprocal fashion with M-protein (I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, submitted). This intriguing finding suggests that by varying the relative amounts of M-protein and EH-myomesin the M-bands can adjust their mechanical properties to the particular requirements in a given muscle type (see below).

The M-band is an important site for targeting of metabolic enzymes and signaling proteins

Several enzymes have been localized in the M-band region: the most prominent is the muscle isoform of creatine kinase, M-CK, which is involved in the restoring of an energy reservoir (Turner *et al.*, 1974; Stolz *et al.*, 1998). However also enolase, phosphofruktokinase and adenylate kinase localize to the M-band, suggesting a general role of the M-band as a targeting site for metabolic enzymes (Keller *et al.*, 2000; Lange *et al.*, 2002).

In addition, several proteins have been identified recently at the M-band that might play a more dynamic role than the other constituents and might be involved in signaling processes between the sarcomere and the nucleus to respond to alterations in physiological requirements. Among these is DRAL/FHL-2, a member of the LIM domain family that is localized in a subregion of the I-band, binds also to titin in the M-band and is probably involved in targeting metabolic enzymes such as M-CK there (Lange *et al.*, 2002). DRAL/FHL-2 possesses four and a half LIM domains

and has been shown to interact with several nuclear factors such as PLZF and androgen receptor; therefore an additional role in nuclear signalling also in cardiomyocytes is very likely (Muller *et al*., 2000; McLoughlin *et al*., 2002).

Several members of muscle specific ring finger proteins have also been localized at the M-band, like e.g. MURF-1 and MURF-2. While their targeting site seems to be in the titin molecule at the edge of the M-band region, they can also translocate to the nucleus following stimulation either by glucocorticoids in the case of MURF-1 or by cellular stress in the case of MURF-2 (McElhinny *et al*., 2002; Pizon *et al*., 2002).

A protein that also might be involved in stress sensing mechanisms is Smpx/Csl. It is localized in the I-band region as well as in the M-band and was originally identified as a protein upregulated following the application of passive stretch on skeletal muscle fibers (Kemp *et al*., 2001; Palmer *et al*., 2001). The largest M-band component that was identified recently, is obscurin, an 800 kDa protein that is expressed exclusively in striated muscle. Obscurin shows a modular composition similar to titin, myomesin and M-protein, consisting mainly of Ig but also several fibronectin type III domains. At its C-terminus obscurin has in addition several sequence domains that have been associated with different signalling pathways, such as an IQ motif for interaction with calmodulin, a GEF domain, and a PD homology domain characteristic for activators of the small GTPase Rho (Young *et al*., 2001).

In conclusion, the M-band region might not be as static as previously thought. In fact, it may be one of the major regions in the sarcomere, where unusual stress on the myofibrils is monitored and a cellular response is elicited by signalling to the nucleus.

Is the M-band connected with the lateral membrane?

At present, it is still not quite clear how the M-bands are integrated with the lateral membrane in a way comparable to the costameres at the Z-disc level, but the existence of such links would be plausible from the viewpoint of the sarcomere stability. Transverse connections from the M-band to the membrane have been visualized in electron micrographs, but their molecular nature remains to be defined (Pierobon-Bormioli, 1981; Wang and Ramirez-Mitchell, 1983). The M-band component skelemin was previously suggested as a linker molecule between the borders of the M-band and the intermediate filaments (Price and Gomer, 1993). However, skelemin is in fact a splice isoform of myomesin (Steiner *et al*., 1999) and has therefore been renamed EH-myomesin (Agarkova *et al*., 2000). Since EH-myomesin is the exclusive isoform of myomesin during early embryonic heart development, presumably integrating thick and elastic filaments in the first sarcomeres, an additional involvement of EH-myomesin in lateral M-band connections is rather unlikely. Recently it has been demonstrated that this link might be provided by spectrin and its associated proteins. Antibody localization experiments for spectrin show striations that overlay the M-bands at the cytoplasmic membrane of skeletal muscle cells (Williams and Bloch, 1999; Flick and Konieczny, 2000). A muscle specific splice isoform of spectrin might be involved in this process (Hayes *et al*., 2000). Further evidence for a spectrin mediated link between the M-band and the membrane comes from data which show that ankyrin, which interacts with erythrocyte spectrin, has also been shown to localize to the membrane in the M-band region and furthermore to interact with the giant M-band component obscurin (Bagnato *et al*., 2003).

Molecular organization of the M-band

Despite significant progress during the last decade, the understanding of the function of the two putative M-band structural components myomesin and M-protein is still incomplete. Biochemical analysis has concentrated on solid phase binding assays, which localize the myosin binding site to the N-terminus of both M-band proteins (schematically depicted in Figure 3, Obermann *et al*., 1997; Obermann *et al*., 1998). The affinity of myomesin to titin (Nave *et al*., 1989) was explained by the interaction of myomesin fragment My4-My6 with titin Ig-domain m4 (Obermann *et al*., 1997). In contrast, the titin-binding site in M-protein is not identified yet, although biochemical assays (Obermann *et al*., 1998) and electron microscopy (EM) data (Obermann *et al*., 1996) indicated the existence of such an interaction. However, the binding assays, performed on recombinant protein fragments, might not reflect the whole complexity of interactions characteristic for eukaryotic cells because neither of these domains but the Ig-domain My2 on its own was found to be essential for the proper targeting of epitope-tagged myomesin fragments to the M-band in cultured cardiomyocytes (Auerbach *et al*., 1999). This finding raises the intriguing question, with which component the second domain of myomesin interacts in the M-band.

A major progress was the development of the current model of M-band organization based on biochemical assays and epitope localization of antibodies directed against defined domains of titin, myomesin and M-protein (Obermann *et al*., 1996). According to this model, the M1 line (see Figure 1) is formed by M-protein molecules arranged mostly perpendicular to the myosin filaments. This explains the fact that the presence of M1 on EM preparations correlates with the expression of M-protein in a given muscle type (Carlsson *et al*., 1990; Pask *et al*., 1994). The major part of myomesin is proposed to run in parallel to the sarcomere longitudinal axis and, together with titin to form the so called M-filaments, i.e. structures running in the half way distance between the thick filaments (see Figure 1). Further, the first three domains of myomesin bend towards the myosin filament and interact with it by their N-terminal domain. Although the predictions of

the model are in good agreement with the electron microscopic data, it fails to answer the most important question, namely how the connection between the thick filaments is accomplished. Since M-protein is absent in many muscle types, myomesin has to be the main protein providing this connection. However, the length of the few domains situated at the N-terminus of myomesin, which connect titin with the neighbouring myosin filaments according to Obermann and colleagues (see Figure 3), is not sufficient to allow for the significant change in lattice spacing in the working sarcomere. Further, the finding of the conserved splicing site between the domains six and seven of conventional myomesin (Agarkova *et al.*, 2000) indicates that this part of the molecule might be implicated in the M-band bridging as well and suggests the presence of another M-band binding epitope on the myomesin C-terminus. This is the issue for future studies.

Distinct M-band composition in different muscles

The recent data on a distinct molecular composition of M-bands in different muscle types (Grove *et al.*, 1985; Grove *et al.*, 1989; Agarkova *et al.*, 2000; I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, paper submitted), can be summarized in a simple diagram and allow conclusions on the functional significance of the individual components.

The different muscle types are arranged along the horizontal axis in Figure 4. The vertical axis shows the relative amount of different M-band components. First, it seems that myomesin (solid line) is the universal M-band component; its total amount in the sarcomere (irrespective of the presence or absence of the EH-fragment in the middle) remains roughly the same in all kinds of striated muscle. It is tempting to assume that the total amount of myomesin is proportionally correlated to the amount of myosin heavy chain (MyHC) in the sarcomere, but this statement needs measurements that are more precise. In contrast, M-protein (dotted line) and the EH-myomesin isoform (dashed line) appear in a reciprocal fashion. Indeed, in the M-bands of the embryonic heart sarcomere all myomesin molecules contain the EH-fragment whereas M-protein is absent. Therefore, the embryonic heart is positioned at the extreme left, while on the other extreme there is fast muscle, which does not have any EH-myomesin in the M-bands, but expresses a maximal level of M-protein. All other muscle types are positioned somewhere in between these extreme cases. The approximate positions, corresponding to slow muscle and adult heart of mouse, are schematically depicted (Figure 4). Species differences are found in these positions, because the slow fibers of rat, for example, express much less EH-myomesin than the ones of mouse (I. Agarkova, unpubl.). These variations in the M-band composition might reflect either the increased shortening velocity of the slow myosin in small animals (Widrick *et al.*, 1997) or a special architecture of mouse hind limb muscles.

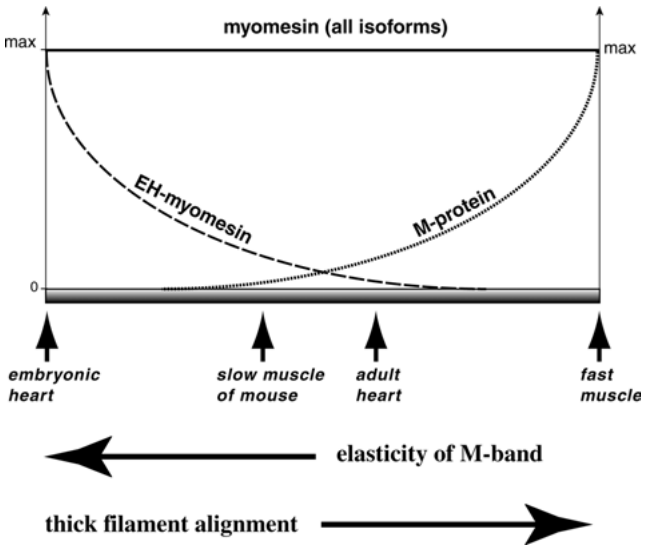

Fig. 4. Distinct molecular composition of the M-band in different muscle types: the vertical axis shows the relative amounts of expression of a given M-band component, while the different muscle types are arranged along the horizontal axis. Myomesin, calculated as a sum of both isoforms (solid line), seems to be proportional to the amount of MyHC in all muscle types. In contrast, M-protein (dotted line) and EH-myomesin (dashed line) seem to be counter-players and appear in a mutually exclusive manner in different muscle types. The curves correspond to the general tendency, since some fibers can co-express small amounts of both components or, alternatively, are devoid of both. The position corresponding to the slow muscle might change in different species.

Possibly, also the different regions of the heart might differ in their M-band composition, particularly during development.

This scenario indicates that myomesin is the fundamental bridging molecule while M-protein and EH-myomesin contribute mutually exclusive properties to the M-band. M-protein might improve the stability of the thick filament lattice in muscles that are characterized by quick force development (Pask *et al.*, 1994) while EH-myomesin might render these connections more loose and elastic (I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, submitted). This suggestion is based on the EM studies of developing embryonic heart, which showed that alignment of thick filaments gradually improves until birth (Smolich, 1995) in direct correlation with the decreasing proportion of EH-myomesin in the M-band (Agarkova *et al.*, 2000).

'Fuzzy' sarcomere hypothesis

What could be the reason for the differential M-band composition in the slow fiber sarcomere as depicted in Figure 4? As discussed above, the M-band filaments provide the precise alignment of thick filaments during sarcomere activation. We suggest that the special M-band protein composition might compensate for increased shearing forces, appearing between neighbouring myosin filaments in the slow fiber sarcomere during the contraction. This idea is supported by the observation that the Z-disc, which is the other transverse

structure in the sarcomere, is also thicker in slow fibers, despite a lower speed and power of contraction (Yamaguchi *et al*., 1985). Moreover, the shift in Z-disc width in slow skeletal muscle (Carlsson and Thornell, 1987) is nicely correlated with the changes in the M-band composition during postnatal development (I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, submitted).

We argue that the increased shearing stresses might originate from higher heterogeneity of actin filament length in the slow fiber sarcomere. In addition to the increased average length of thin filaments in slow fibers (Granzier *et al*., 1991), they might be more variable within a sarcomere. The variability of actin length in different fiber types was not studied systematically until recently due to ambiguities in the EM-fixation procedures (Page and Huxley, 1963). A recent study avoided these problems by measuring thin filament lengths by deconvolution of fluorescent images (Littlefield and Fowler, 2002). The analysis of these data indicates that actin length variations increase in the following sequence: fast muscle-slow muscle-embryonic heart, which is in perfect agreement with the relative proportion of the EH-myomesin isoform in these muscles (see Figure 4). The mechanism of actin filament length regulation in the sarcomere is not clear, but it was suggested that nebulin (Kruger *et al*., 1991) together with the actin filament capping protein, tropomodulin, plays a role in it (Gregorio *et al*., 1995; McElhinny *et al*., 2001). Recently, it was found that slow and fast muscle in chicken express different tropomodulin isoforms, probably regulating the degree of actin filament length variability (Almenar-Queralt *et al*., 1999).

Slow and fast muscles express also different titin isoforms, while in all cases the slow soleus sarcomeres express the longer splice variant than the ones in fast fibers. The slow fiber titin contains more Z-repeats allowing the formation of wider Z-discs (Sorimachi *et al*., 1997). The M-band portion of titin in soleus and diaphragm muscle contains an additional exon, Mex-5 (Kolmerer *et al*., 1996). The secondary structure of this fragment, as well as its function is not clear at present, but potentially it may contribute to the special M-band design in the slow fiber sarcomere.

Because M-band filaments and the elastic part of titin might co-operate during muscle contraction (see above), some correlation between their isoform content in different muscle types can be expected. Indeed, the I-band portion of titin in soleus muscle, which also contains the longer EH-myomesin isoform in the M-bands (I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, submitted), is much more compliant in comparison to the one from fast psoas fibers (Horowits, 1992). This is achieved by the addition of more Ig-modules as well as a longer PEVK segment (Freiburg *et al*., 2000). The significance of this phenomenon is not clear yet. However, it was found that the Ig-domains that are added to the titin elastic region in soleus muscle are characterized by a lower level of unfolding force than the Ig-domains from the constitutively spliced region (Watanabe *et al*., 2002). This suggests that the longer PEVK domain as well as some alternatively spliced titin Ig-domains might play the role of a buffer during the eccentric contraction. These domains might be more dynamic, with a lower threshold for the force responsible for unfolding but also displaying faster recovery. This might prevent the damage on the more tightly folded Ig-domains from the constitutively expressed region.

The features of the slow fiber sarcomere, mentioned above, are summarized in Figure 5. While the classical sarcomere in fast muscle shows an almost crystalline packing of contractile filaments, the slow fiber sarcomere is noticeably less well ordered; therefore, we named it 'fuzzy' sarcomere (Figure 5). The actin filaments in the slow fiber sarcomere have a more irregular length, leading to a fuzzy border of the actin filament zone, which is not easily distinguishable on EM-pictures. The Z-discs are wide and the M-bands are more elastic and therefore are barely discernible in EM-pictures, e.g. 'fuzzy' as well (I. Agarkova, R. Schoenauer, E. Ehler and J.C. Perriard, submitted). In addition, this type of sarcomere contains a more compliant titin isoform. What is the benefit of such adaptations? The activation in the stretched state (eccentric contraction) is very dangerous for muscles and can lead to the destruction of their contractile structures. It is likely that the special construction of 'fuzzy' sarcomeres in slow fibers renders them more stable under conditions that correspond to the descending limb of the sarcomere length-tension curve. This assumption is supported by data showing that the slow fibers are affected less than the fast ones in muscles of a mixed fiber composition subjected to eccentric contraction (Friden and Lieber, 1998; Vijayan *et al*., 2001). This was explained by an increased number of sarcomeres per unit length in the slow vs. fast fibers (Brockett *et al*., 2002). However, only intra-sarcomeric protection mechanisms can explain an improved stability of the slow fibers to eccentric contraction measured on skinned myofibers with normalised number of sarcomeres (Macpherson *et al*., 1996).

We argue that all mentioned structural adaptations of the slow sarcomere might favour an increase of its working range. Indeed, it is known that unlike the fast fibers, the slow fibers of the hind limb are active during all phases of locomotion (Walmsley *et al*., 1978). Thus, they have to function safely and efficiently in both compressed and extended state. In contrast, the fast fibers produce short and powerful efforts by strictly defined positions of the limb. Moreover, the prolonged periods of activity of slow fibers promote the development of the regions of irregular sarcomere length (Morgan, 1990) and therefore threaten their stability. Probably, this has to be compensated by the reinforcement not only of the intra-sarcomeric but also of the extra-sarcomeric stabilizing systems. This hypothesis is supported by observations that the relative content of

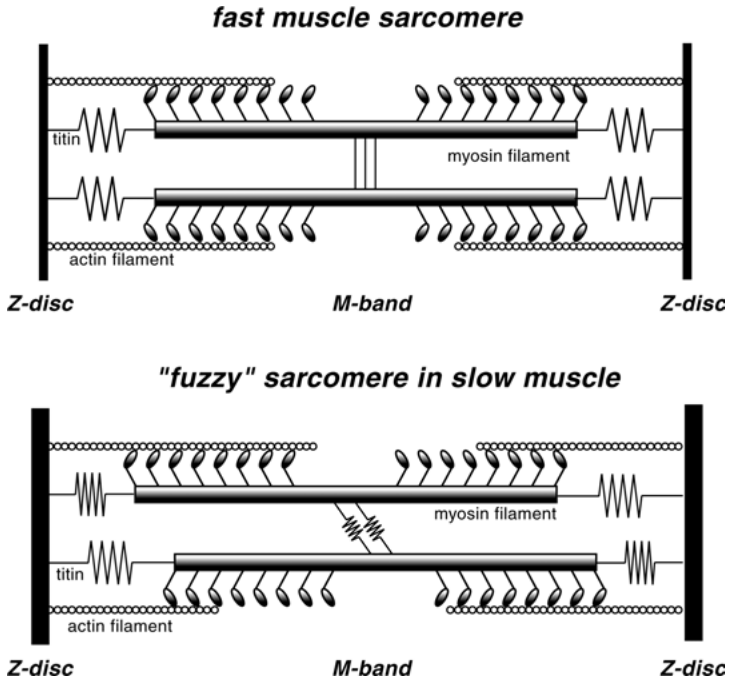

Fig. 5. Schematic drawing highlighting the main differences between the fast and the slow fiber sarcomere: the classical sarcomere in fast muscle demonstrates the perfect alignment of both contractile elements, actin and myosin filaments. It is equipped with thin Z-discs and a short titin isoform. In contrast, the slow fiber sarcomere shows less order in the alignment of contractile elements; therefore, we named it 'fuzzy'. In this sarcomere the length of the actin filaments is more variable, and the M-band bridges are more elastic. The extensible portion of titin is longer and more compliant. All these adaptations might improve the stability of the slow fiber sarcomere during prolonged activity over a broader working range.

desmin (Chopard *et al.*, 2001), dystrophin (Ho-Kim and Rogers, 1992) and other components of the dystrophin glycoprotein complex (Chopard *et al.*, 2000) is significantly higher in slow soleus vs. fast EDL muscle. Probably because of this, the passive tension increases more steeply in slow than in fast fibers (Granzier *et al.*, 1991; Mutungi and Ranatunga, 1996), which is even more surprising considering the expression of the very compliant titin isoform in slow fibers (Freiburg *et al.*, 2000). Thus, the stability of the slow fiber sarcomere might be controlled by two different mechanisms: first, by the special 'fuzzy' design of the sarcomere itself that provides a smoother transition to the descending limb of the force-length relationship and, second, by the faster increase of the stiffness of stabilizing cytoskeletal filaments that prevent an extensive elongation of individual sarcomeres. Summarizing the facts mentioned above, it appears that the construction of fast fibers is optimized to produce the most powerful contraction, whereas the slow fibers are optimized for better reliability during continuous contractile activity.

What happens to the sarcomere if the M-band disappears?

A mouse lacking one of the putative M-band bridging molecules myomesin or M-protein has not been generated yet. Therefore, the effect of an absence of the M-band components on sarcomeric structure and stress maintenance is unknown so far. However, recently a mouse model was published that comes close to a deletion of the organized M-band structure. A conditional knock-out of the titin M-band region, by removing the exons Mex1 and Mex2 leads to sarcomeric disassembly in both heart and skeletal muscle, and finally to death of the animal (Gotthardt *et al.*, 2003). The authors suggested that the absence of the titin kinase domain and the binding site for MURF-1, a novel titin-binding protein, deregulate the myofibrillar signaling and result in the decay of the contractile apparatus. However, according to the current M-band model, the deleted part of the titin molecule includes also the binding epitopes for the major M-band bridging molecules, myomesin and M-protein (Obermann *et al.*, 1997). Unfortunately the expression and localization of myomesin or M-protein were not investigated in this mouse model yet; however based on the lack of electron dense M-bands on the published EM pictures, an absence or delocalization of these bridging molecules is rather likely. Thus, this mouse model provides an opportunity to check what happens to a sarcomere lacking proper M-band organization.

There is evidence that the presence of M-band proteins is crucial for the completion of the sarcomere cytoskeleton during myofibrillogenesis, since these proteins might be involved in the formation of a hexagonal lattice of thick filaments (Ehler *et al.*, 1999). Accordingly, Gotthardt *et al.* (2003) found that deletion of the M-band region of titin in the heart during early development leads to embryonic death. However, if this part of titin was deleted after birth, mutant animals died only a few weeks later, due to sarcomeric disorganization and muscle weakness.

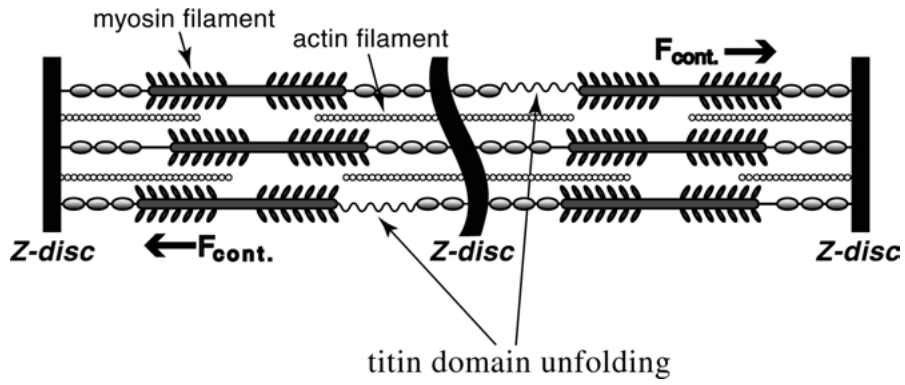

Fig. 6. Consequences of the absence of the M-band for sarcomere stability: for simplicity, the elastic portion of titin is shown as a series of folded domains. After activation, the myosin filaments will accidentally deviate from the central position and stretch some of the titin filaments. This pulling force might cause the denaturation of some parts of the titin molecules. This effect might accumulate and lead to a progressive wearing out of titin in muscles, subjected to prolonged periods of contractile activity. As a result, the degree of thick filament misalignment will progressively increase, as well as the stress that is applied to the Z-discs. According to this model, the following symptoms are to be expected in sarcomeres subjected to multiple contractions: A-band misalignment, Z-disc deformation, and overall sarcomere widening followed by sarcomere disassembly.

To explain these data, the effect of the repeated activation on a sarcomere without the M-band must be considered. The schematic model of such a sarcomere is shown in Figure 6: the activation displaces thick filaments that lack M-band stabilization in different directions, depending on the random difference in the numbers of activated cross-bridges on each side. Some of the thick filaments might accidentally deviate so far, that the titin filaments, whose stiffness is adjusted to A-band positioning in the presence of intact M-band connections, might not succeed to bring it back to the center of the sarcomere during the short period of muscle relaxation. Consequently, upon the next activation the degree of thick filament deviation will grow, increasing the stretch of the titin molecules, which are attached to this filament.

As discussed above, stretch enhances the probability of titin Ig-domain unfolding (Rief *et al.*, 1998). Therefore, if the contraction continues, eventually one of the Ig-domains from the stretched titin filament might unfold. In contrast to passively stretched muscles, where unfolding events randomly happen in some titin molecules without fatal consequences (Minajeva *et al.*, 2001), in this case the damage will accumulate and this presents a clear danger. As discussed above, the short period of relaxation in continuously working muscles might be not sufficient for complete recovery of titin molecules. To compare, the mouse heart beats with the rate of up to 10 times per second, whereas the refolding of titin Ig-domains might need seconds (Carrion-Vazquez *et al.*, 1999). Once happened the unfolding event in one of titin strands favours the weakening of the neighbouring titin molecules that are attached to the same thick filament, thereby leading to a vicious cycle of destruction. Due to the gradual increase in contour length of some of the titin filaments, the Z-discs might be exposed to increased shearing stress (Figure 6). According to this model, continuously active muscles, such as cardiac muscle or muscles responsible for posture, will be damaged first. One might expect sarcomere widening, misalignment of the thick filaments, ruptured Z-discs and finally a gradual sarcomeric disassembly. Indeed, exactly these effects were observed in the muscles of the M-band titin mutant mice (Gotthardt *et al.*, 2003). This hypothetical model shows that, if the M-band, an important element of the sarcomeric stabilization system, is lacking, the continuous contraction might threaten sarcomere integrity. We argue that a thorough study of the dynamics of the pathological process in the different muscles of titin M-band mutant mice is needed to elucidate this issue.

Conclusion

The focus of this review was how to maintain the stability of the muscle sarcomere. We emphasized that apart from providing maximally effective contraction and force transduction, the sarcomeric cytoskeleton functions to prevent the contractile machinery from self-destruction. Several filament systems provide such a protection and the investigation of their relative importance will be subject of future studies.

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